Telomerase activity in primary cultures of normal adrenocortical cells

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Abstract

Telomerase activity was measured in isolated cells from bovine and human adrenal cortex, in cells in primary culture, in cells in later passages in culture, and in cells genetically modified by expression of hTERT (human telomerase reverse transcriptase). Telomerase activity in freshly isolated bovine adrenocortical cells and in human adrenal cells from donors of various ages (6–79 years) was very low or undetected. However, primary bovine adrenocortical cell cultures were strongly positive for telomerase activity, and primary human adrenocortical cell cultures were weakly positive. Both cell types proliferate in primary culture but proliferation of bovine cells is much more vigorous. When primary bovine cells were subcultured to make successively secondary and tertiary cultures, telomerase activity declined strongly, and was undetected

by the third passage. There was only a slight decrease in growth rate over this period. Levels of the telomerase RNA component did not change with passage number when assessed by semi-quantitative competitive RT-PCR. When both bovine and human cells were infected with a retrovirus encoding hTERT, telomerase activity in the cells was very high. We conclude that in the adrenal cortex, as in some other tissues, TERT expression is regulated and upregulation of telomerase activity is associated with rapid proliferation in primary culture. Telomerase activity is not maintained, and introduction of TERT is required for stable telomerase activity and for immortalization.

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Introduction

Most normal somatic cells from humans and other mammals cannot divide indefinitely because of the progressive loss of DNA from telomeres, the specialized ends of the linear chromosomes (Harley *et al.* 1990, Allsopp *et al.* 1992). Shortening of telomeres eventually results in a permanent cell cycle block (replicative senescence) (Greider 1990, Harley 1991, Smith & Pereira-Smith 1996). The gradual erosion of telomeres during repeated cell division results from the combined effects of the inability of normal DNA replication to completely extend telomeric DNA and the lack of expression of the telomerase reverse transcriptase (TERT) component of the telomerase ribonucleoprotein complex (Lingner *et al.* 1997, Meyerson *et al.* 1997). Age-related telomere shortening, resulting from repeated cell division in the absence of TERT, has been observed in many human tissues (Hornsby 2001). The consequences of this loss of telomeric DNA are limitations on the reserve capacity of tissues to undergo cell proliferation when required. The repression of TERT expression appears to be an anti-cancer mechanism, because telomerase activity is required for full neoplastic conversion of normal cells (Hahn *et al.* 1999).

Therefore, TERT repression is an example of antagonistic pleiotropy; there is a trade off between the benefits (protection against cancer in early life) versus problems (exhaustion of proliferation in tissues in old age) (Hornsby 2001). Telomere shortening, leading to replicative senescence, has also been described in bovine cells in culture (Thomas *et al.* 2000), and bovine cells *in vivo* undergo age-related telomere shortening (Lanza *et al.* 2000).

Stem cells and germ line cells, which normally express TERT and have telomerase activity, are able to fully replicate telomeric DNA and thereby are able to divide indefinitely (Thomson *et al.* 1998, Pittenger *et al.* 1999). Whereas cells that do not have telomerase activity are able to divide only a limited number of times before they senesce, in cells with forced expression of TERT the progressive shortening of telomeres is prevented; such cells are termed ‘immortal’ because they continue to proliferate indefinitely (Bodnar *et al.* 1998, Kiyono *et al.* 1998).

In previous experiments from this laboratory we investigated telomerase activity and telomere maintenance in bovine adrenocortical cells. Clones of normal bovine adrenocortical cells undergo loss of telomeric DNA in culture and eventually enter replicative senescence (Thomas *et al.* 2000). Similarly, human adrenocortical cells

have a finite proliferative potential in culture (Hornsby *et al.* 1992) and this is also associated with telomere shortening (Yang *et al.* 2001). When bovine adrenocortical cells were transfected with a combination of plasmids encoding human TERT (hTERT) (under the control of the CMV promoter) and SV40 T antigen (under the control of the SV40 promoter), telomere length was stabilized, cells showed a high level of telomerase activity, and were apparently immortalized (Thomas *et al.* 2000). Cells transfected only with the SV40 T antigen plasmid did not show telomerase activity and were not immortalized. hTERT-modified clones could be transplanted into adrenalectomized *scid* mice and rescued the animals from the lethal effects of adrenalectomy by the formation of functional adrenocortical tissue (Thomas *et al.* 2000). Both human and bovine adrenocortical cells show telomere length stabilization and apparent immortalization following infection with a retrovirus encoding hTERT (Yang *et al.* 2001). In summary, previous experiments have shown that normal bovine adrenocortical cell clones and genetically modified clones not transfected with hTERT suffer telomere shortening, are telomerase-negative, and have a finite proliferative potential, whereas both bovine and human adrenocortical cells expressing hTERT have stabilized telomere lengths and are apparently immortalized.

However, studies on telomerase activity and telomere length regulation have shown that it is an oversimplification to classify cell types simply as having a finite life span (TERT expression repressed) or immortal (TERT expressed, or cells prevent telomere shortening by alternative means). Some normal human cells, including stem cells and other cells that are required to undergo repeated replications, do express TERT, but this expression is tightly regulated, by processes not yet well understood (Hodes 1999). In keratinocytes from skin and uterine cervix, and in several types of endothelial cells, telomerase activity is detected when cells are first isolated from the body and stimulated to divide in culture (Yasumoto *et al.* 1996, Hsiao *et al.* 1997, Kunitura *et al.* 1998). Longer term proliferation is associated with a loss of telomerase activity. Long-term cultures of normal human cells lack sufficient telomerase activity for telomere maintenance, with the important exceptions of embryonic stem cells and mesenchymal stem cells (Thomson *et al.* 1998, Pittenger *et al.* 1999).

In the present experiments we investigated whether adrenocortical cells may have regulated telomerase activity. We measured telomerase activity in primary tissue and primary cultures of adrenocortical cells of both bovine and human origin.

Materials and Methods

Cell and tissue preparation

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion from the adrenal cortex of

Figure 1 Growth of primary bovine adrenocortical cells in culture and of cells in subcultures. For assessment of cell growth cells were plated in 24-well plates. The arrows indicate subcultures using a 1:5 split ratio.

2-year-old steers, as previously described (Hornsby & McAllister 1991). Tissue fragments were dissociated to cell suspensions using enzymatic and mechanical dispersal (3 h incubation with 1 mg/ml type I-A collagenase and 0.1 mg/ml DNase, both from Sigma Chemical Co., St Louis, MO, USA). Primary cell suspensions were stored frozen in liquid nitrogen. Frozen cells were thawed and replated in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% heat-inactivated horse serum and 0.1 ng/ml recombinant basic fibroblast growth factor. This was supplemented with 1% v/v Ultraser G (Biosera, Villeneuve-la-Garenne, France), a mixture of growth factors, as previously described. The gas phase used was 90% N₂, 5% O₂ and 5% CO₂. For preparation of cells for telomerase assays, cells were grown in 10-cm culture dishes. For cell proliferation measurements, cells were grown in 24-well plates. Cells were trypsinized and counted using a Coulter counter.

Human adrenal glands were obtained from kidney organ donors or from patients undergoing resection of the kidney for renal neoplasms. We are grateful to the staff of Lifegift, Houston, TX, USA, for their assistance in providing adrenals from kidney transplants. Tissue fragments were dissociated as described for bovine adrenocortical cells. Telomerase activity was measured on cell suspensions that had not been cultured. Additionally, cells were plated and grown in culture as described for bovine adrenocortical cells, except that we used collagen I-coated tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA).

Figure 2 Telomerase activity in bovine adrenocortical cells. 1°, 2° and 3° indicate cells harvested from confluent primary, secondary and tertiary cultures of bovine adrenocortical cells (cells harvested at the times indicated by the arrows in Fig. 1). hTERT=bovine adrenocortical cells infected with a retrovirus encoding hTERT. The amount of protein (μ g) used per reaction in the TRAP assay is indicated above the lanes. 3T3=positive control (3T3 cells); control=no cells control; i.c.=internal control for amplification by PCR.

For retroviral infection, plasmids encoding retroviruses derived from mouse Moloney leukemia virus were transfected into the PT67 mouse packaging cell line (Clontech, Palo Alto, CA, USA), which produces a virus type (10A1) that efficiently infects most human cells (Miller & Chen 1996) and also bovine adrenocortical cells (T Suwa, L Yang & P J Hornsby, unpublished observations). pBabe-hTERT, in which hTERT is expressed from the retroviral long terminal repeat, was a gift from Dr J Campisi, (Lawrence Berkeley National Laboratory, Berkeley, CA, USA) (Kim *et al.* 1999) and was constructed from pCI-neo-hTERT (Counter *et al.* 1998). Plasmid pLEGFP-N1, encoding a retrovirus in which green fluorescent protein (GFP) is under the control of an internal CMV promoter, was obtained from Clontech. For retroviral infection of adrenocortical cells, cells were plated in 10-cm Transwell dishes (Corning, Cambridge, MA, USA). The producer cells were plated in the insert, placing the producer cells immediately above the target cells, separated by a polycarbonate membrane with 3- μ m pores. The infection was

allowed to take place over 4 days, followed by selection in puromycin or G418. After antibiotic selection over 7–10 days, the cells were grown to a sufficient cell number for biochemical analysis.

Measurement of telomerase activity by telomerase repeat amplification protocol (TRAP) assay

Cell pellets were extracted with 0.5 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β -mercaptoethanol, 10% glycerol and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) on ice for 30 min (Kim *et al.* 1994). Cell lysates were clarified by centrifugation at 14 000 g for 30 min at 4 °C. The TRAP assay was performed on the supernatants using the TRAP-EZE kit (Intergen, Purchase, NY, USA). Two microliters of cell extract were mixed with 5 μ l 10 $\times$ TRAP reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, and

Figure 3 Telomerase activity in human and bovine adrenocortical cells. hTERT=human or bovine adrenocortical cells infected with a retrovirus encoding hTERT; GFP=human or bovine adrenocortical cells infected with a retrovirus encoding GFP; 1 µg protein was used per reaction in the TRAP assay. 3T3=positive control (3T3 cells); control=no cells control; i.c.=internal control for amplification by PCR.

10 mM EGTA), 1 µl 50 × modified dNTP mix, 2 µl TS primer, 1 µl modified primer mix, 0.4 µl (4 µCi) [α - 32 P]dCTP, 0.4 µl Taq polymerase (ExTaq; Takara, Kyoto, Japan) and 38.2 µl water. The incubation was performed at 37 °C for 30 min. PCR was then performed (94 °C for 30 s, 59 °C for 30 s for 29 cycles). Products (20 µl) were separated by 10% PAGE. Gels were dried and exposed to X-ray film for appropriate lengths of time.

Semi-quantitative competitive RT-PCR

Levels of expression of telomerase RNA component (TERC) were assessed by semi-quantitative competitive

RT-PCR using β -actin as a control mRNA. RNA was extracted from cultured cells using RNazol (Tel-Test, Friendswood, TX, USA) in accordance with the manufacturer's instructions. Three micrograms of total RNA were included in a 20 µl reverse transcriptase reaction comprising 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM of each dNTP, 10 pmol random hexamer primers, and 200 units reverse transcriptase (Superscript II; Invitrogen, Baltimore, MD, USA) at 42 °C for 60 min. The first-strand cDNA products (2 µl) were used in a PCR reaction in which [α - 32 P]dCTP was incorporated. Preliminary experiments showed that both β -actin and TERC were amplified to

Figure 4 Telomerase assays performed on cell suspensions (without culture) obtained from adrenal glands of donors of the indicated age and sex. hTERT=human adrenocortical cells infected with a retrovirus encoding hTERT; 1 µg protein was used per reaction in the TRAP assay. 3T3=positive control (3T3 cells); control=no cells control; i.c.=internal control for amplification by PCR.

suitably convenient levels by adding the TERC primers first, adding the β -actin primers after five cycles, and then allowing the reaction to proceed for another 20–26 cycles. Primers specific for bovine TERC (100 pmol) were added and the PCR reaction was started by the addition of 2.5 units Taq polymerase (Promega, Madison, WI, USA). After five cycles, primers specific for bovine β -actin (5 pmol) were added and the reaction was allowed to proceed to a total of 25–31 cycles (95 °C/60 °C/72 °C). The reaction products (10 µl) were separated by PAGE. Gels were dried and exposed to a Phosphorimager screen (Molecular Dynamics, Amersham Pharmacia Biotech, Newark, NJ, USA) for quantitation of ^{32}P incorporated into the β -actin and TERC products (1074 and 395 bp respectively).

Results

We investigated telomerase activity in primary cultures of bovine adrenocortical cells and in subsequent passages. In

the standard method for culturing these cells, the primary cells dissociated from the adrenal gland are plated at a relatively low density and are allowed to grow for 7–10 days to confluence; they are then serially subcultured at a 1:5 split ratio to generate secondary, tertiary and subsequent cultures. Almost all primary cells enter DNA synthesis after a lag of approximately 48–72 h, and then undergo very rapid growth in the primary culture. The growth rate declines slightly as the cells are passaged, although it stabilizes and is then relatively constant for a large number of population doublings until the cells enter senescence (Hornsby *et al.* 1992). Figure 1 shows the rate of growth in primary, secondary and tertiary cultures of bovine adrenocortical cells.

Telomerase activity was readily detected and relatively high in cells in primary culture (Fig. 2). It became weaker in the secondary culture and was faintly detected in the tertiary culture. In cells from subsequent cultures it was undetected. Primary bovine adrenocortical cells were also infected with a retrovirus encoding hTERT, and were harvested following selection in puromycin. As expected,

Figure 5 Levels of the TERC in bovine adrenocortical cells assessed by semi-quantitative competitive RT-PCR. 1°, 2° and 3° indicate RNA from cells harvested from confluent primary, secondary and tertiary cultures. RNA samples were used for RT-PCR as described in Materials and Methods. In the PCR step, [α - 32 P]dCTP was included in the reaction. On the left is an autoradiogram of PCR products specific for bovine β -actin and TERC, produced by 28 PCR cycles. On the right, radioactivity in these products was measured by Phosphorimager and has been plotted for the three passages (primary (●), secondary (■) and tertiary cultures (▲)) as a function of the number of PCR cycles.

these cells had high telomerase activity, relatively higher than that of cells from the primary culture. Telomerase activity remained high in hTERT-modified cells when they were grown for ~ 50 more population doublings, whereas it was undetected in any nongenetically modified bovine adrenocortical cells grown to a population doubling level beyond that equivalent to tertiary cultures.

Figure 3 shows that primary human adrenocortical cell cultures were also weakly telomerase-positive. Telomerase activity was readily detectable when cells had been infected with a retrovirus encoding hTERT, and weakly detectable in cells infected with a control retrovirus encoding GFP. Telomerase became undetected in normal and GFP-expressing cells grown for ~ 10 more population doublings. For comparison, Fig. 3 also shows bovine adrenocortical cells after infection and antibiotic selection with the GFP-encoding retrovirus. Note that the autoradiographic exposure time used in this figure is longer than that used in Fig. 2; the telomerase activity in the

GFP-expressing bovine adrenocortical cells is at the limit of detection. Longer-term growth of these cells produced telomerase-negative cultures, whereas both human and bovine hTERT-modified cells were stably telomerase-positive.

These results show that for both bovine and human cells there is detectable telomerase activity in primary cultures which disappears after cells are grown for longer periods. We investigated whether there was any detectable telomerase activity in adrenocortical tissue before cell culture. Figure 4 shows that in a variety of cells isolated from adrenal glands of donors of different ages there was no detected telomerase activity. Similarly, no telomerase activity was detected in fresh bovine adrenocortical tissue before cell culture.

The decline in telomerase activity in early passages of bovine adrenocortical cells appeared to be caused by loss of expression of TERT, based on evidence presented here that ectopic expression of TERT in later passages restored

telomerase activity. Although it was not possible to measure bovine TERT mRNA directly, we were able to assess levels of TERC, the telomerase RNA component, using semi-quantitative competitive RT-PCR. Figure 5 shows that the ratio of TERC RNA to β -actin mRNA assessed by this method did not differ among primary, secondary and tertiary cultures after 25, 28 or 31 PCR cycles.

Discussion

In these experiments we found that both bovine and human adrenocortical cells were telomerase-positive in primary cultures, and passaged cultures at low population doubling levels, but became telomerase-negative when proliferation continued. Retroviral introduction of the hTERT gene greatly increased telomerase activity in both human and bovine cells. hTERT protein is apparently able to cooperate with bovine TERC RNA as well as with human TERC RNA, although the somewhat lower telomerase activity in hTERT-modified bovine cells suggests a lower activity of the hTERT/bovine TERC RNA ribonucleoprotein complex than the hTERT/human TERC RNA complex. Most cell types express TERC RNA, even though TERT is not expressed (Feng *et al.* 1995, Tsao *et al.* 1998).

Telomerase activity was higher in primary bovine adrenocortical cells than in human adrenocortical cells. It has been suggested that low levels of telomerase activity in normal cells is associated with cell division (Belair *et al.* 1997). Bovine cells divide at a much higher rate than human adrenocortical cells. Human adrenocortical cell proliferation is dependent on donor age (Yang *et al.* 2001), but even fetal human adrenocortical cells do not match the rate of proliferation of primary bovine adrenocortical cells. Nevertheless, telomerase activity was weakly detected in primary human adrenocortical cells. Although the level of telomerase activity correlates with growth rate in primary cells, the later disappearance of telomerase activity cannot simply be the result of decreased growth. Cells were harvested for TRAP assays when most cells were not dividing (see Fig. 1). The percentage of cells in division was approximately the same in primary, secondary and tertiary bovine adrenocortical cells, but telomerase activity differed greatly.

The decline in telomerase activity in culture is currently unexplained. The decrease observed in epithelial cells was attributed to the loss of a stem cell population (Kunimura *et al.* 1998), but this has not been rigorously demonstrated. In the case of adrenocortical cells, dilution of a minority of telomerase-positive cells by overgrowth of telomerase-negative cells seems unlikely. There is relatively little dilution of the cells from the primary to the tertiary culture, and little cell death. If the decline results from dilution, the number of telomerase-positive cells must be

small and they must be more susceptible to cell death than other cells in the population. It seems more likely that TERT expression is regulated and that environmental conditions in the primary culture stimulate TERT expression, by increased transcription or other means. If so, the stimulus appears to be lost as cells continue to grow in longer-term cultures.

In conclusion, these data add to a growing body of evidence that telomerase activity is under stringent specific regulation in many cell types. Elucidation of the factors that regulate telomerase activity in primary cultures would clearly be of both practical and theoretical importance.

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